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Short Communication

3-Iodo-4-aminoquinoline derivative sensitises resistant strains of *Plasmodium falciparum* to chloroquine

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ABSTRACT

Chloroquine (CQ), the first cost-effective synthetic antimalarial, is rendered ineffective in malaria-endemic regions owing to the rise and spread of CQ-resistant *Plasmodium falciparum*. In this report, we show that a halogen derivative of CQ, namely 3-iodo-CQ, inhibits the proliferation of CQ-sensitive and -resistant *P. falciparum* in a verapamil-insensitive manner. Similar to CQ, the antimalarial activity of 3-iodo-CQ is likely due to its inhibition of β-haematin formation. Interestingly, the presence of non-inhibitory concentrations of 3-iodo-CQ potentiated the antiproliferative activity of CQ against CQ-resistant strains or *P. falciparum* transfectants expressing wild-type or mutant *P. falciparum* CQ resistance transporter (PfCRT) ($C2^{GC03}$ or $C4^{Dd2}$, respectively). These findings demonstrate that halogenation of the third position of 4-aminoquinoline, with a simple one-step reaction from CQ, generates a novel derivative that is active against CQ-sensitive and -resistant *P. falciparum*, possibly by inhibiting the activity of mutant PfCRT.

1. Introduction

Malaria remains a major cause of mortality among children aged <5 years in developing countries [1]. Chloroquine (CQ), used for decades to treat malaria, has been all but abandoned due to the rise and spread of CQ-resistant *Plasmodium falciparum* in malaria-endemic regions [2].

One strategy to develop antimalarial drugs has focused on chemical modification of quinoline-based drugs, with known targets and mechanism of action, in an effort to reduce the cost and time of drug development [3]. Several aminoquinoline derivatives were shown to be effective against CQ-sensitive (CQ^S) and CQ-resistant (CQ^R) parasites [4,5].

CQ inhibits parasite proliferation by binding to free haemin in the digestive vacuole (DV) and interfering with haemozoin formation [6]. Polymorphisms in the *P. falciparum* CQ resistance transporter (PfCRT) mediate parasite resistance to CQ [7]. A key mutation in PfCRT (e.g. K76T), seen frequently in CQ^R parasites, allows the transport of protonated CQ from the parasite's digestive vacuole DV [8]; however, the mechanism of CQ transport by mutant PfCRT remains unresolved [9]. In this report, we describe the synthesis and characterisation of a halogen derivative of CQ modified at the third position of 4-aminoquinoline. The antimalarial activities of the 3-iodo-CQ and its effects on different strains of *P. falciparum* or wild-type and mutant PfCRT transfectants are described.

2. Materials and methods

2.1. Synthesis and characterisation of 3-halo-chloroquine derivative

N-iodosuccinimide (1.5 equivalents) and CF_3CO_3H (0.5 equivalents) were dissolved in 5 mL of acetonitrile and were heated to reflux for 30 min. CQ (1 equivalent) was then added and the mixture was refluxed at 80 °C for an additional 2 h. After cooling, the reaction mixture was evaporated to dryness, was dissolved in water and was then neutralised by aqueous NaOH. The mixture was extracted with CH_2Cl_2 (3 × 50 mL) and the extracts were dried with $MgSO_4$. After removal of the solvent, the residue was chromatographed on silica gel [eluent volumes: hexane/ethyl acetate/triethylamine (7:2.5:0.5) and hexane/ethyl acetate/triethylamine/MeOH (7:2.5:0.5:0.25)] to give the corresponding free base of the 3-halo-CQ in good yield (see Appendix: Supplementary methods for a full description of the synthesis and characterisation of 3-iodo-CQ). All chemicals, unless indicated otherwise, were of the highest available grade and were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2. Culture conditions and the effects of drugs on parasite proliferation

CQ^S (3D7, D10 and $C2^{GC03}$) and CQ^R (K1, Dd2 and $C4^{Dd2}$) *P. falciparum* strains were maintained in culture in RPMI 1640 medium supplemented with 2 mM L-glutamine, 25 mM HEPES, 50 mg/L (w/

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v) hypoxanthine, 2.5 μg/mL gentamicin (Thermo Fisher Scientific, Waltham, MA) and 5% (v/v) human serum (A + type pooled; Interstate Blood Bank, Chicago, IL) essentially as previously described by Trager and Jensen [10]. To measure the effects of drugs on the proliferation of parasites, increasing concentrations of CQ or 3-iodo-CQ without or with verapamil (as indicated in Tables 1 and 2) were added to ring-stage cultures at 0.5% parasitaemia, 2% haematocrit and cultures were allowed to proliferate for 72 h at 37 °C. Plates were frozen at –80 °C, then thawed in the presence of lysis buffer [20 mM Tris (pH 7.5), 5 mM ethylene diamine tetra-acetic acid (EDTA), 0.008% saponin, 0.08% Triton X-100 and 0.2 μL/mL of 10 000 × SYBR nucleic gel stain dye; Thermo Fisher Scientific] as previously described [11]. Results were analysed using GraphPad Prism v.5.0 (GraphPad Software Inc., La Jolla, CA) to obtain the 50% inhibitory concentration (IC_{50}). All graphs shown represent the mean ± standard deviation (S.D.) of three independent experiments performed in triplicate.

2.3. β-Haematin inhibition assay

The β-haematin inhibitory activity assay was used to determine the IC_{50} value for CQ and 3-iodo-CQ as described previously [11]. The relative absorbance (OD) was measured at 405 nm using a Synergy™ H4 hybrid multimode microplate reader (BioTek Instruments, Thermo Fisher Scientific). The results are expressed as percentage inhibition of β-haematin formation [% inhibition = (1 – (OD drug)/(OD control)) × 100]. Data were analysed using GraphPad Prism v.5.0 to obtain the IC_{50} value. All graphs shown represent the mean ± S.D. of three independent experiments performed in triplicate.

3. Results and discussion

The synthetic strategy for the 3-iodo-CQ derivative utilised a radical halogenation reaction of CQ with *N*-halo succinimides that allowed for the preparation of the free base in excellent yields (Fig. 1A). The antimalarial activity of the 3-iodo derivative relative to CQ was evaluated in vitro. Fig. 1B shows the proliferation of CQ^S (3D7 and D10) and CQ^R (Dd2 and K1) strains of *P. falciparum* in the presence of increasing molar concentrations of CQ or 3-iodo-CQ derivative. Modification of the third position of the 4-aminoquinoline caused a ca. 10-fold drop in the IC_{50} value of 3-iodo-CQ relative to unmodified CQ. The IC_{50} values for 3-iodo-CQ and CQ for the four different strains of CQ^S (3D7 and D10) and CQ^R (Dd2 and K1) parasites were ca. 300–700 nM and 22–180 nM, respectively. These results are consistent with an earlier finding whereby modification of the 7-chloro-4-aminoquinoline ring of CQ reduced its antimalarial activity [12], whilst changes to the CQ side chain enhanced the activity [13].

CQ is thought to bind ferriprotoporphyrin IX (FP-IX), mainly in the DV, and to interfere with the ability of the parasite to detoxify the released haemin into insoluble haemozoin or β-haematin [14]. Given the structural similarities between CQ and 3-iodo-CQ, we examined the ability of 3-iodo-CQ to inhibit β-haematin formation in vitro, as previously demonstrated for CQ [15]. Our results show that both CQ and 3-iodo-CQ inhibited β-haematin formation, albeit at different molar concentrations, with IC_{50} values for CQ and 3-iodo-CQ at 8 mM and 12 mM, respectively (see Fig. S1). The ability of CQ to interfere with haemozoin formation is governed by two chemical moieties: (i) the 7-chloro group, which plays a crucial role in the inhibition of haemozoin formation; and (ii) the presence of basic heterocycles and amino groups in its side chain that allow for the accumulation of the quinoline drug in the parasite's DV [12].

Verapamil has been shown to reverse CQ resistance in vitro by blocking CQ transport from the DV of parasites expressing mutant PfCRT [16]. Given the results in Fig. 1B, it was of interest to investigate the ability of verapamil to increase the sensitivity of CQ^R parasites to 3-iodo-CQ. To test the latter possibility, the proliferation of CQ^S and CQ^R strains was determined in the presence of CQ or 3-iodo-CQ with or without 1 μM verapamil. As expected, the presence of verapamil caused a significant increase in the sensitivity of CQ^R (Dd2) but not CQ^S (3D7) strains to CQ (Fig. 1C); however, verapamil did not modulate the sensitivity of CQ^S or CQ^R strains to 3-iodo-CQ (Fig. 1C). These results suggest that 3-iodo-CQ is not transported via wild-type or mutant PfCRT. However, given the presence of other genetic differences between the two strains (3D7 and Dd2), in addition to mutations in PfCRT, it was not possible to rule out the role of PfCRT in modulating the sensitivity of the parasite to 3-iodo-CQ. To determine the role of PfCRT on the susceptibility of the parasite to 3-iodo-CQ, we made use of two clones generated from the CQ^S parental strain GC03 that harbour either the wild-type *pfcrt* allele ($C2^{GC03}$) or a mutant Dd2 *pfcrt* allele ($C4^{Dd2}$) [17]. Presence of the Dd2 *pfcrt* allele in the CQ^S strain $C2^{GC03}$ did not modulate the IC_{50} values of 3-iodo-CQ (175.3 nM and 168.7 nM for $C2^{GC03}$ and $C4^{Dd2}$, respectively). Moreover, the presence of verapamil did not modulate the sensitivity of $C4^{Dd2}$ expressing mutant PfCRT to 3-iodo-CQ, whilst it significantly increased its sensitivity to CQ (see Fig. S2). Together, these results demonstrate that 3-iodo-CQ, unlike CQ, is not a substrate for wild-type or mutant PfCRT. However, given that both CQ and 3-iodo-CQ inhibit β-haematin formation, it was of interest to determine whether 3-iodo-CQ potentiates the toxicity of CQ against CQ^S and CQ^R parasites. Indeed, 3-iodo-CQ potentiated the antiproliferative effect of CQ on 3D7 or D10 (CQ^S) and Dd2 or FCR3 (CQ^R) *P. falciparum* using three non-inhibitory doses of 3-iodo-CQ (50, 100 and 200 nM) (Table 1). The fact that the IC_{50} value of both drugs in combination is lower than the IC_{50} value of each drug alone suggests a chemosensitisation or reversal effect. To determine the nature of the interaction between CQ and 3-iodo-CQ, an isobologram plot based on the sum of the fractional inhibitory concentrations (FICs) derived from IC_{50} values for each of the compound alone and in combination was used to predict synergistic interactions (concave curve), an antagonistic interaction (convex curve) or no interaction (a straight line) [18]. The results show moderate synergy for CQ and 3-iodo-CQ against Dd2 (CQ^R strain) with a mean FIC_{index} of 0.7, whilst no synergy was observed between CQ and 3-iodo-CQ against 3D7 with a mean FIC_{index} of 1.06 (see Fig. S3). The increased sensitivity of 3D7 and D10 to CQ in the presence of non-inhibitory concentrations of 3-iodo-CQ is interesting and may be due to either of the following: (i) wild-type PfCRT allows the transport of CQ; or (ii) 3-iodo-CQ potentiates the antiproliferative effect of CQ via another mechanism. To examine the effect of PfCRT mutations on the ability of 3-iodo-CQ to enhance the potency of CQ, *pfcrt*-modified clones ($C2^{GC03}$ with wild-type *pfcrt* allele and $C4^{Dd2}$ with mutant Dd2 *pfcrt* allele) were chosen for their common isogenic background with the only modification being in the *pfcrt* allele [17]. Table 2 shows the proliferation of parasites in the presence of CQ at the IC_{50} (23.34 ± 4.45 nM and 198.95 ± 7.42 nM for $C2^{GC03}$ and $C4^{Dd2}$, respectively) and fixed concentrations for both 3-iodo-CQ and verapamil (25, 50, 75, and 100 nM). Verapamil was used at a higher concentration (e.g. 1000 nM) to demonstrate clear potentiation of CQ sensitivity in this clone based on earlier observations (unpublished data). The results show that 3-iodo-CQ and verapamil did not potentiate the effect of CQ on the proliferation of $C2^{GC03}$ clone expressing wild-type PfCRT (Table 2). However, by contrast, 3-iodo-CQ demonstrated a significant synergy with CQ on the proliferative capacity of $C4^{Dd2}$ expressing the mutant PfCRT (Table 2). It is important to note that 100 nM 3-iodo-CQ was more effective than 1000 nM verapamil (Table 2). The above results suggest that 3-iodo-CQ may be a clinically useful compound, in combination with CQ, as antimalarial therapy against CQ^R *P. falciparum*. To assess the effect 3-iodo-CQ on mammalian cell growth, two human cell lines [T-cell lymphoma (CCRF-CEM) and cervical epithelial carcinoma (HeLa) cells] were cultured in increasing concentrations of CQ or 3-iodo-CQ (0–50 μM). Neither CQ nor 3-iodo-CQ caused significant inhibition